# Inhibition of *Salmonella enterica* by Plant-Associated Pseudomonads In Vitro and on Sprouting Alfalfa Seed†

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## ABSTRACT

Foodborne illness due to the consumption of contaminated raw or lightly cooked sprouts is a continuing food safety concern. In this study, we tested several plant-associated pseudomonads for their ability to inhibit the growth of *Salmonella enterica* both in vitro and in situ. An agar spot bioassay method was used with three different media. Only *Pseudomonas fluorescens* 2-79 produced clear zones of inhibition when tested against five serovars of *S. enterica,* and activity was dependent on media type and serovar. The antibiosis by derivative strains of *P. fluorescens* 2-79 defective in the production of phenazine-1-carboxylic acid and fluorescent siderophore was not reduced, indicating that these known antimicrobial metabolites were not responsible for the inhibition observed in our studies. However, mutants defective in the regulatory gene *gacS* (global antibiotic and cyanide control) were severely reduced in inhibitory activity. In tryptic soy broth, the control cultures of a cocktail of *S. enterica* strains reached approximately 10 log CFU/ml by 24 h but, when coinoculated with *P. fluorescens* 2-79, reached only approximately 5 log CFU/ml. The addition of *P. fluorescens* 2-79 to the seed soak water prior to the germination of alfalfa seed previously inoculated with a cocktail of *S. enterica* strains led to an average reduction of 5 log CFU/g at 6 days of sprouting without an adverse effect on sprout yield or appearance. Time course studies indicated that *S. enterica* outgrowth was controlled on days 1 through 6 of sprouting. Competitive exclusion as a potential food safety intervention for seed sprouts merits further study.

During the past decade, the consumption of raw or lightly cooked seed sprouts contaminated with *Salmonella enterica* or *Escherichia coli* O157 has been responsible for at least 27 outbreaks in the United States, resulting in more than 1,600 reported cases of foodborne illness *(8, 44, 51).* Sprouts are one of the produce items most often associated with outbreaks of salmonellosis *(41).* Because of the food safety concerns with sprouts, numerous studies on the use of various chemical and physical methods for eliminating bacterial human pathogens from sprout seed have been carried out *(1, 8).* In contrast, there have been few studies on the use of biological agents to control the outgrowth of human pathogens from contaminated sprout seed *(25, 32, 56).* There is a great deal of published information on the biopreservation of foods with natural microflora, such as lactic acid bacteria *(42).* Biological control has also been intensively studied as a means of controlling plant diseases since at least the early 1920s *(3),* including the use of fluorescent pseudomonads as antagonists *(11, 18).* More recently, the use of competing microorganisms (in a technique often referred to as competitive exclusion) to inhibit the growth and survival of human pathogens in a variety of animals has been studied *(38, 49).* Several commercial biological-based products are on the market worldwide for use in controlling the colonization of newly hatched chicks with *Salmonella (38).*

Bacterial human pathogens are surprisingly good competitors on sprout surfaces when present initially on seed, and they can grow to high levels (from $10^6$ to $10^7$ CFU/g) on sprouting alfalfa seed *(19).* However, an indication that competitive exclusion can occur naturally on alfalfa sprouts was evidenced by the small amount of growth these pathogens showed (1 log or less) when inoculated onto sprouts 1 to 5 days into the sprouting process *(5, 39).* Thus, the key to effective control may be in ensuring the presence of high levels of effective competitive microflora on the seed at the time of initial germination.

In this study, we determined the ability of several plant-associated fluorescent pseudomonads to inhibit *S. enterica* both in vitro and in situ on sprouting alfalfa seed. The majority of the strains tested are known biological control agents with activity against a variety of soilborne fungal plant diseases *(36).* Fluorescent pseudomonads represent a major proportion of the population of native bacteria on sprouting seed *(2, 24, 26, 33)* and thus appear well adapted to growth on sprout surfaces. Initial studies on the mode of action of the most antagonistic bacterium identified in this study, *Pseudomonas fluorescens* 2-79, are also included.

## MATERIALS AND METHODS

**Microbial strains.** The plant pathogenic fungus *Gaeumannomyces graminis* var. *tritici* ATCC 28229 was obtained from the American Type Culture Collection (Manassas, Va.). The source

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TABLE 1. *Bacterial strains used in this study*

| Bacterium | Serovar or strain | Description[a] | Source and/or reference(s)[b] |
|---|---|---|---|
| *Bacillus amyloliquefaciens* | | | ERRC culture collection |
| *Salmonella enterica* | Anatum F4317 | Sprout-related outbreak, sprout isolate | P. Griffin, CDC, Atlanta, Ga. |
| | Infantis F4319 | Sprout-related outbreak, sprout isolate | P. Griffin |
| | Muenchen HERV2C | Sprout-related outbreak, seed isolate | W. Fett |
| | Newport H1275 | Sprout-related outbreak, clinical isolate | P. Griffin |
| | Stanley H0558 | Sprout-related outbreak, clinical isolate | P. Griffin |
| *Pseudomonas aureofaciens* | 30-84 | $PCA^+$, 2-OH-$PCA^+$, 2-OH-$PHZ^+$ | *28, 34* |
| | 30-84Z | $Phz^-$ (*phzB*::*lacZ* genomic fusion) | *34* |
| | ATCC 13985 | $PCA^+$, 2-OH-$PCA^+$, 2-OH-$PHZ^+$ | ATCC, *28* |
| *P. chlororaphis* | ATCC 9446 | $PCA^+$ | ATCC, *28* |
| *P. fluorescens* | 2-79 (NRRL-15132) | $PCA^+$, $Flu^+$ | *10, 31, 47* |
| | 2-79LacZ | $PCA^-$ (*phzD*::*lacZ* genomic fusion), $Flu^+$ | L. Thomashow, USDA-ARS, Pullman, Wash. |
| | 2-79.59.34 | 2-79$RN_{10}$::Tn5, $Km^r$, $PCA^+$, $Flu^-$ | *13, 31* |
| | 2-79.59.34.24 | 2-79$RN_{10}$::Tn5, $Km^r$, $PCA^-$, $Flu^-$ | *13, 31* |
| | 2-79B46 | 2-79$RN_{10}$::Tn5, $Km^r$, $PCA^-$, $Flu^+$ | *31, 47* |
| | CHA0 | $DAPG^+$, $PLT^+$, $PRN^+$, $HCN^+$ | *6, 43, 53* |
| | Pf-5 | $DAPG^+$, $PLT^+$, $PRN^+$, $HCN^+$ | *15, 55* |
| | Q2-87 | $DAPG^+$, $HCN^+$ | *20, 35* |
| | Q8r1-96 | $DAPG^+$, $HCN^+$ | *37* |
| *P. marginalis* | ATCC 10844 | Pectolytic | ATCC, *29* |

[a] PCA, phenazine-1-carboxylic acid; 2-OH-PCA, 2-hydroxyphenazine-1-carboxylic acid; 2-OH-PHZ, 2-hydroxyphenazine; Phz, phenazines; Flu, fluorescent pyoverdin siderophore; $Km^r$, kanamycin resistant; DAPG, 2,4-diacetylphloroglucinol; PLT, pyoluteorin; PRN, pyrrolnitrin; HCN, hydrogen cyanide.

[b] ERRC, Eastern Regional Research Center; CDC, Centers for Disease Control and Prevention; ATCC, American Type Culture Collection; USDA-ARS, U.S. Department of Agriculture, Agricultural Research Service.

and description of the bacterial strains used in this study are listed in Table 1. *P. fluorescens* 2-79.59.34.24 and 2-79B46 have mutations in the global regulatory gene *gacS (46)*. For long-term storage, bacteria were kept at −80°C in tryptic soy broth (TSB; BBL, Becton Dickinson, Sparks, Md.) supplemented with 20% glycerol (Sigma, St. Louis, Mo.). Frozen stock cultures of the pseudomonads were streaked on *Pseudomonas* agar F (PAF; BBL, Becton Dickinson) with or without kanamycin (Sigma), as appropriate, 1 day before the start of each experiment, with incubation at 28°C. For the culturing of resistant strains (2-79.59.34, 2-79.59.34.24, and 2-79B46), kanamycin was added to PAF for a final concentration of 100 μg/ml (PAF+K). *S. enterica* serovars Anatum F4317, Infantis F4319, Newport H1275, and Stanley H0558 were adapted to grow in the presence of streptomycin sulfate (final concentration, 1,000 μg/ml; Sigma) and nalidixic acid (sodium salt; final concentration, 100 μg/ml; Aldrich Chemical, Milwaukee, Wis.) by standard procedures. Strains of *S. enterica* were maintained on tryptic soy agar (TSA; BBL, Becton Dickinson) with or without the addition of antibiotics, as appropriate, at 4°C, with monthly transfers to fresh agar.

**Test for protease activity.** Litmus milk agar (BBL, Becton Dickinson) plates were inoculated in duplicate with *P. fluorescens* strain 2-79 wild type and its derivatives. The plates were incubated at 28°C for 48 h and then observed for zones of clearing that surrounded bacterial growth, indicative of protease activity.

**Test for pectolytic activity.** Pectolytic activity was tested for according to Liao *(23)* with semisolid pectate medium (pH 7.1) supplemented with casamino acids (0.3%; BBL, Becton Dickinson). The experiment was performed twice.

**Test for antifungal activity.** To confirm that the strains of *Pseudomonas* were producing antimicrobial compounds with antifungal activity as previously reported, the antifungal activity of each potential antagonistic bacterial strain was tested against *G. graminis* var. *tritici* on potato dextrose agar (BBL, Becton Dickinson) according to the method of Weller et al. *(54)*. Each strain was tested three times in independent experiments.

**Agar spot bioassays.** Initially, a deferred agar spot bioassay method and a simultaneous method were compared, with *P. fluorescens* 2-79 as the producer strain, *S. enterica* serovar Stanley H0558 as the indicator strain, and Luria broth (LB; Invitrogen Life Sciences, Paisley, Scotland) solidified by the addition of 15 g/liter of Bacto agar (LA; BBL, Becton Dickinson) as the growth medium. Each culture dish (100 by 15 mm) used for the agar spot bioassays contained 25 ml of media. For the simultaneous method,

uninoculated plates of LA were overlaid with 6 ml of molten water agar (Bacto agar, 6%; BBL, Becton Dickinson) inoculated with 60 μl of an overnight culture (approximately 9 log CFU/ml) of *S. enterica* serovar Stanley H0558 that had been grown in TSB (37°C, with shaking at 250 rpm). After the overlay had solidified, 10 μl of an overnight broth culture (TSB, 28°C, 250 rpm) of *P. fluorescens* 2-79 was spotted (two spots per plate per strain) on the seeded agar surface, along with 10 μl of uninoculated TSB as a control. Plates were incubated at 28°C, with readings taken at 24 and 48 h. For the deferred method, strain 2-79 was spotted onto LA, with incubation for 48 h at 28°C. After incubation, bacterial cells were killed by a 1-h exposure to chloroform vapors under a chemical fume hood. Each plate was then overlaid with a suspension of *S. enterica* serovar Stanley H0558 prepared in water agar as described above. After the overlaid medium had solidified, the plates were incubated at 37°C. Zones of inhibition were measured from the outer edge of the producer colony to the outer edge of the inhibition zone for the indicator strain after 24 and 48 h of incubation. The experiments were performed three times

On the basis of the information obtained by the initial results, all *Pseudomonas* strains were then tested by the deferred method, as stated above, for the production of antimicrobial substances, with activity against five indicator strains represented by five *S. enterica* serovars on LA as well as on the additional agar media PAF and malt agar (MA; final pH of 5.3; Sigma). Duplicate plates were spotted with each *Pseudomonas* strain (one spot per strain per plate) per *S. enterica* serovar per media type for each experiment. When LA and MA were used, incubation was for 48 h before overlaying. For PAF, incubation was for 24 h. Inhibition was recorded 24 and 48 h after overlaying with *S. enterica,* as stated above. Deferred agar spot bioassays were also carried out as described above for selected *Pseudomonas-Salmonella* strain combinations on the following media: LA and MA prepared in 100 mM morpholinepropanesulfonic acid buffer, pH 6.8 (Sigma) (final pH of 6.8); LA and PAF supplemented with $FeCl_3 \cdot 6H_2O$ (100 μM; J. T. Baker Chemical Co., Phillipsburg, N.J.) (final pH of 6.7 and 7.0, respectively); and LA supplemented with glucose (20 g/liter, final pH of 6.2; Sigma). Experiments were replicated independently at least three times.

**Disk diffusion antibacterial tests.** A filter paper disk diffusion test by the agar overlay method *(27)* was conducted to test a commercial preparation of phenazine-1-carboxylic acid (PCA; Chembridge Corporation, San Diego, Calif.) for antibacterial activity against *S. enterica* serovar Anatum F4317. PCA was dissolved at a concentration of 1 mg/ml in methylene chloride and tested at up to 100 μg per disk. Methylene chloride (100 μl) alone was added to disks as a control. The experiment was carried out two times, with duplicate assay plates per bacterium for each experiment.

**Heat inactivation of antimicrobial factor(s) produced on agar media.** The effect of heating on the antimicrobial activity of *P. fluorescens* 2-79 on agar media was tested. *P. fluorescens* 2-79 was spot inoculated onto four plates of LA (one spot per plate) and incubated for 48 h at 28°C; then, the bacteria were killed with chloroform vapors. Half of the plates were kept at 75°C for 30 min to inactivate heat-labile inhibitory factors, and the other half (unheated controls) remained at room temperature. The colonies of *P. fluorescens* 2-79 were then overlaid with *S. enterica* Stanley H0558 as described above. After the agar overlays had solidified, plates were incubated at 37°C. Zones of inhibition were measured after 24 and 48 h of incubation. The experiment was carried out two times.

**Tests for bacteriophage activity.** The ability to transfer the inhibitory agent(s) from clear zones of inhibition formed during agar spot bioassays to a fresh lawn of indicator bacteria was tested by stabbing the inhibition zone with a sterile wooden toothpick and then immediately stabbing a freshly prepared lawn of the same indicator bacterium overlaid onto the same agar medium at several locations. Lawns were observed for the formation of clear inhibition zones (plaques) after incubation at 37°C for up to 48 h. Alternatively, agar plugs (1.5-mm diameter) were aseptically cut from clear inhibition zones and crushed in 100 μl of 100 mM potassium phosphate buffer, pH 7.4. Then, 10 μl of chloroform was added with mixing, and the suspension was centrifuged to separate the aqueous and chloroform layers. Fifty microliters of the top aqueous layer was carefully removed, and serial 10-fold dilutions were prepared in sterile distilled water. Undiluted and diluted samples were spotted (10 μl) onto a freshly prepared lawn of the appropriate indicator strain, with incubation at 37°C. Plates were observed at 24 and 48 h for the formation of plaques.

**Production of antimicrobial compound(s) in broth and heat stability.** *P. fluorescens* 2-79 and its derivatives 2-79LacZ, 2-79.59.34, 2-79.59.34.24, and B46 were grown on PAF or PAF+K, as appropriate, at 28°C for 48 h. A single colony of each strain was used to inoculate 10 ml of LB contained in 125-ml flasks, one flask per strain. Flask contents were shaken (250 rpm) at 28°C for 48 h. The cultures were subjected to centrifugation (10,000 × *g*, 15 min, 4°C), and the supernatant fluids were collected; then, each sample was filtered through a 0.45-μm sterile filter (Millex-GV, Millipore Corporation, Bedford, Mass.). For use as the indicator strain, *S. enterica* serovar Anatum F4317 was grown overnight (18 to 20 h) in 10 ml of LB at 37°C with shaking (250 rpm). Three hundred microliters of the overnight culture was suspended in 20 ml of molten (50°C) LA, and the molten agar was poured onto a culture plate (100 by 15 mm). After cooling, three equidistant wells were cut from each plate with a sterile #3 cork borer (6-mm diameter). To two of the wells, 50 μl of cell-free culture supernatant was added. To the third well, 50 μl of LB alone was added as a negative control. Plates were then incubated at 37°C and observed for the formation of inhibition zones surrounding the wells at 24 and 48 h. A similar experiment was performed with *P. fluorescens* 2-79 both as the producer strain and as the indicator strain. Duplicate bioassay plates were used per experiment per strain, and the experiments were replicated once.

Cell-free supernatants from strain 2-79 were also subjected to heating in a boiling water bath for 10 min. After allowing the treated supernatants to cool to room temperature, both heated and nonheated cell-free supernatants, along with LB alone as a negative control, were added to the wells, and the experiment was continued as described above. The experiment was performed three times.

**Competitive inhibition with respect to the growth of *S. enterica* in broth culture.** *P. fluorescens* 2-79 and its derivatives 2-79LacZ, 2-79.59.34, 2-79.59.34.24, and 2-79B46 were tested for their ability to inhibit the growth of a cocktail of the five *S. enterica* strains in TSB following procedures similar to those of Zhao et al. *(57).* The *P. fluorescens* strains were cultured in TSB overnight (28°C, 250 rpm). Then, the cells were harvested by centrifugation, washed once with sterile purified water, and recentrifuged. The final cell pellets were suspended in sterile purified water to give an optical density at 600 nm ($OD_{600}$) of 0.10 (approximately 8 log CFU/ml). The five *S. enterica* serovars used as indicators for the agar spot bioassays described above were prepared in the same manner, except that the five overnight cultures

were combined before harvesting. The suspensions of the individual *P. fluorescens* strains were diluted 10-fold, and the suspensions of the *S. enterica* cocktail were diluted 10,000-fold. One milliliter of the pseudomonad culture was added to 10 ml of TSB contained in a 125-ml flask to give a starting population of approximately 5 log CFU/ml; similarly, 1 ml of the pathogen cocktail was added to give a starting population of approximately 3 log CFU/ml. One flask was inoculated with the *Salmonella* cocktail alone as a control. The flasks were incubated with shaking (250 rpm) at 24°C, and samples were removed to prepare serial decimal dilutions in sterile 0.1% peptone water at 0, 6, and 24 h of incubation. Dilutions were plated in triplicate on TSA (for total bacterial counts—*Pseudomonas* plus *S. enterica*) and XLT-4 (BBL, Becton Dickinson) (for *S. enterica*). Preliminary experiments indicated that *P. fluorescens* strain 2-79 did not grow on XLT-4 after 24 h of incubation at 37°C. The inoculated TSA plates were incubated at 28°C for 48 h, and the XLT-4 plates were incubated at 37°C for 24 h before colonies were counted. Subtraction of the *S. enterica* population (as determined on XLT-4) from the total bacterial populations (as determined on TSA) equalled the population of the fluorescent pseudomonad strains.

**Growth inhibition of *S. enterica* on sprouts.** Commercial alfalfa seed for sprouting was purchased from the Caudill Seed Company (Louisville, Ky.). To prepare the seed inoculum, the four antibiotic-resistant strains of *S. enterica* were used to inoculate single tubes containing 2 ml of TSB, and the cultures were incubated with shaking (250 rpm) at 37°C for 18 to 20 h. Starter cultures (0.1 ml each) were then used to inoculate 25 ml of TSB, and the cultures were incubated as stated above. Samples (1 ml) from each of the four flasks were combined, and the remainder of each was used to determine the pathogen populations of the individual cultures by preparing serial decimal dilutions with sterile 0.1% peptone water and plating in triplicate onto TSA plus streptomycin (1,000 μg/ml) and nalidixic acid (100 μg/ml) (TSASN). Bacterial cells in the combined samples were pelleted by centrifugation, and the cell pellets were washed once with sterile 0.1% peptone water. Then, the washed cells were recentrifuged, and the final pellet was taken up in 2 ml of sterile 0.1% peptone water. Alfalfa seed (400 g) was placed in a sterile stomacher bag with a nylon-mesh liner. Sterile distilled water (200 ml) and 40 μl of inoculum were added to the bag. The remainder of the procedure, including a determination of the initial inoculum load on the seed, was performed as previously described *(7)*. The inoculated seed was stored at 4°C until use.

The bioassays were carried out in small glass vials similar to the experiments conducted by Matos and Garland *(25)*. The *Pseudomonas* strains to be tested as antagonists were cultured at 28°C overnight. Single colonies were used to inoculate 2 ml of TSB, and the cultures were incubated at 28°C for 18 to 20 h with shaking (250 rpm). The cells were harvested by centrifugation, washed once with sterile distilled water, and recentrifuged. The final cell pellets were suspended in sterile distilled water to give an $OD_{600}$ of 0.10 (approximately 8 log CFU/ml). For each antagonist strain to be tested, seven seeds previously inoculated with the cocktail of *S. enterica* serovars were placed in 2 ml of the antagonist inoculum and left to sit at room temperature on the laboratory bench for 2 h. As negative controls, two sets of seven inoculated seeds were each placed in 2 ml of sterile distilled water. After 2 h, seeds were transferred to sterile glass vials (17 ml) lined at the bottom with two wetted disks of filter paper (Whatman no. 2, Whatman, Maidstone, UK). The bioassay vials were left to sit on the laboratory bench at room temperature (22 ± 1°C), and additional sterile distilled water was added as required.

After 6 days of incubation, all plant materials were aseptically removed from each vial, and the materials were gently blotted dry between sheets of paper towels and weighed. Each sample was then homogenized with a sterile hand-held glass tissue grinder in the presence of 2 ml of sterile 100 mM potassium phosphate buffer, pH 6.8. Serial decimal dilutions were prepared in sterile 0.1% peptone water, and 0.1 ml of undiluted or diluted sample was plated in triplicate onto TSASN. Plates were kept at 37°C, and colonies were counted after 24 and 48 h of incubation. Selected colonies were confirmed as *S. enterica* by slide agglutination tests with *Salmonella* O antiserum Poly A-I and Vi (BBL, Becton Dickinson). The CFU per gram data for the two separate controls of each experiment were averaged, and these values were used to determine the log reductions obtained for the antagonists. Each potential antagonist was tested in three separate experiments.

Subsequently, time course studies with *P. fluorescens* strain 2-79 as the antagonist were carried out. The procedure was the same as stated above with the following modifications: 10 seeds carrying *S. enterica* per vial were used instead of seven; sampling was performed on days 0 (immediately after the 2-h soak period), 1, 2, 3, and 6; on day 0, the entire 2-ml sample was plated (250 μl per plate) for all samples; and on days 1, 2, 3, and 6, the undiluted samples from the pathogen-inoculated seeds treated with strain 2-79 were plated in quadruplicate (250 μl per plate). The experiment was repeated three times.

**Statistical analysis.** Data were subjected to an analysis of variance to determine differences due to treatments on bacterial populations or population reductions (log CFU per gram). Each response was further separated by mean separation tests by means of the Bonferroni least significant difference approach to determine significant differences ($P < 0.05$) *(30)*.

## RESULTS

**Bacterial phenotype and characterization.** The lack of siderophore production by *P. fluorescens* 2-79.59.34 and 2-79.59.34.24 was confirmed by culturing on PAF for 24 h at 28°C and then observing the growth under UV light (365 nm). *P. fluorescens* 2-79LacZ and *Pseudomonas aureofaciens* 30-84Z were confirmed to produce β-galactosidase after growth on LA supplemented with 0.2% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 24 to 48 h at 37°C, whereas the parental strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 were negative for β-galactosidase activity. *P. fluorescens* strains 2-79, 2-79LacZ, and 2-79.59.34 were positive for protease activity, whereas strains 2-79.59.34.24 and 2-79B46 were negative.

None of the fluorescent pseudomonads showed pectolytic activity when tested for the formation of pits on semisolid pectate agar media, except for the positive control strain *Pseudomonas marginalis* ATCC 10844. All strains tested as potential antagonists in this study produced antimicrobial substances, as confirmed by the use of an in vitro antifungal assay with the fungus *G. graminis* var. *tritici* as the test organism, with the exception of *P. fluorescens* strains 2-79LacZ and 2-79B46 as well as *P. aureofaciens* 30-84Z.

**Agar spot bioassays.** Initial results indicated that the antibiosis exhibited by strain *P. fluorescens* 2-79 against *S. enterica* serovar Stanley H0558 on LA was most evident by use of the deferred method, with large clear zones of

TABLE 2. *Inhibition of* Salmonella enterica *serovars on agar media by* Pseudomonas fluorescens *2-79 and derivatives*

| *P. fluorescens* producer strain | Indicator strain | Inhibition ratings[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | LA | LA+G | LA+Fe | PAF | PAF+Fe | MA |
| 2-79 | Stanley H0558 | C (11) | C (10) | C/T (7) | T/− (8) | ND[b] | C/T (6) |
| | Anatum F4317 | C (20) | C/T (14) | C/T (13) | T (7) | C (8) | C (17) |
| | Infantis F4319 | C (17) | C/T (11) | C/T (6) | T (5) | C (6) | C/T (8) |
| | Muenchen HERV2C | C/T (8) | C/T (4) | C/T (6) | T (6) | ND | T (7) |
| | Newport H1275 | C/T (4) | T (4) | T (4) | T/− (8) | ND | T (6) |
| 2-79LacZ | Stanley H0558 | C (13) | C/T (9) | C/T (6) | C/T (2) | ND | C/T (6) |
| | Anatum F4317 | C (20) | ND | ND | C (10) | C (9) | C/T (26) |
| | Infantis F4319 | C (18) | ND | ND | C (7) | C (7) | C/T (9) |
| | Muenchen HERV2C | C (10) | ND | ND | T/− (9) | ND | T (7) |
| | Newport H1275 | C/T (5) | ND | ND | T/− (10) | ND | T (6) |
| 2-79.59.34 | Stanley H0558 | C (9) | C/T (8) | C/T (3) | — | ND | C/T (3) |
| | Anatum F4317 | C (20) | ND | ND | C/T (3) | C/T (4) | C/T (16) |
| | Infantis F4319 | C (16) | ND | ND | T/− (2) | C/T (2) | T (8) |
| | Muenchen HERV2C | C/T (7) | ND | ND | — | ND | T (6) |
| | Newport H1275 | C/T (3) | ND | ND | T/− (2) | ND | T (4) |
| 2-79.59.34.24 | Stanley H0558 | — | ND | ND | — | T/− (16) | T/− (16) |
| | Anatum F4317 | T (1) | ND | ND | — | ND | T/− (16) |
| | Infantis F4319 | — | ND | ND | — | ND | C/T/− (16) |
| | Muenchen HERV2C | — | ND | ND | — | ND | — |
| | Newport H1275 | — | ND | ND | — | ND | C/T (21) |
| 2-79B46 | Stanley H0558 | T (1) | ND | ND | — | ND | C/T/− (13) |
| | Anatum F4317 | C/T (6) | ND | ND | — | ND | T/− (10) |
| | Infantis F4319 | T (3) | ND | ND | — | ND | C/T/− (14) |
| | Muenchen HERV2C | T (1) | ND | ND | — | ND | — |
| | Newport H1275 | — | ND | ND | — | ND | C/T (17) |

[a] LA, Luria agar; LA+G, Luria agar supplemented with glucose (20 g/liter); LA+Fe, Luria agar supplemented with $FeCl_3 \cdot 6H_2O$ (100 μm); PAF, *Pseudomonas* agar F; PAF+Fe, *Pseudomonas* agar F supplemented with $FeCl_3 \cdot 6H_2O$ (100 μm); MA, malt agar; C, clear zones of inhibition; C/T, mixed results with clear or turbid zones; C/T/−, mixed results ranging from no inhibition to clear or turbid zones; T, turbid zones; T/−, mixed results with turbid zones or no inhibition; —, no zones of inhibition. Numbers in parentheses are the average distances (millimeters) of the zone of inhibition from the edge of the producer colonies where inhibition was noted for at least three separate experiments.

[b] Not determined.

inhibition extending outward (an average of 11 mm from the edge of the colonies) (Table 2). When it was grown simultaneously on LA with incubation at 28°C, clear inhibition zones were produced for only 13 of the 20 areas where *P. fluorescens* 2-79 was spotted, and the inhibition zones were quite small, extending 2 to 3 mm from the edge of the *Pseudomonas* colonies. The spotting of TSB alone as a negative control did not cause inhibition. Thus, all further agar spot bioassays were performed by the deferred method.

By the deferred method, all *Pseudomonas* strains were screened for the inhibition of five serovars of *S. enterica* on three different agar media (LA, PAF, and MA). Only *P. fluorescens* 2-79 and some of its derivative strains gave clear zones of inhibition. The data for these strains are presented in Table 2. The degree of antibiosis observed was dependent on the agar media type and the particular *S. enterica* serovar tested. The results presented are for readings taken 24 h after overlaying the plates with the indicator strains, as no significant changes in inhibition were noted after an additional 24 h of incubation. Strain *P. fluorescens* 2-79 and its derivatives 2-79 LacZ (deficient in phenazine production) and 2-79.59.34 (deficient in siderophore production) showed the greatest degree of antagonism. For these strains, the most consistent inhibition occurred after growth on LA, and the most susceptible indicator strain was *S. enterica* serovar Anatum. The addition of glucose (2%) to LA reduced inhibition slightly. The addition of $FeCl_3$ (100 μM) to LA also caused a slight reduction in inhibition, but the addition of $FeCl_3$ to PAF caused an increase in inhibition (Table 2). In contrast, the two *P. fluorescens* 2-79 derivatives with mutations for the production of GacS (2-79.59.34.24 and 2-79B46) demonstrated little-to-no activity. Buffering LA by the presence of 100 mM morpholinepropanesulfonic acid buffer (final media pH of 6.8) had no effect on the antibiosis exhibited by *P. fluorescens* 2-79 against *S. enterica* serovar Stanley H0558 (data not shown).

When clear zones of inhibition were produced by *P. fluorescens* 2-79, 2-79LacZ, and 2-79.59.34, a few isolated colonies of *S. enterica* were always evident in the inhibition zones after 24 to 48 h of incubation. Representative colonies were picked from the clear inhibition zones, purified by single-colony cloning three times on agar media, and then subjected to biochemical reactions with a BBL Enterotube II (BBL, Becton Dickinson) and Gram-Negative Identification cards of the Vitek AMS Automicrobic system

(bioMérieux Vitek, Inc., Hazelwood, Mo.) as well as by slide agglutination tests with *Salmonella* O antisera Poly A-I and Vi (BBL, Becton Dickinson). All of the apparent resistant colonies tested were confirmed to be *Salmonella* by these three methods. When four of the apparent resistant variants of *Salmonella* were retested as indicator strains with *P. fluorescens* 2-79 as the producer strain, no zones of inhibition were noted, which confirmed the resistance of these isolates to the antimicrobial compound(s) produced.

*P. fluorescens* 2-79 did not give inhibition zones when tested against itself as the indicator strain after growth on LA for 48 h before overlaying.

**Sensitivity to PCA.** *S. enterica* serovar Anatum F4317 was not inhibited by the presence of PCA at up to 100 μg per disk. The presence of PCA at 13 μg per disk or greater resulted in hazy zones of inhibition when tested against the sensitive control strain of *Bacillus amyloliquefaciens*. The control disks wetted with methylene chloride alone were not surrounded by inhibition zones.

**Inactivation of inhibitory substances by heat on agar plates.** The heating of LA bioassay plates (at 75°C for 30 min) containing colonies of *P. fluorescens* 2-79 before overlaying them with *S. enterica* serovar Stanley H0558 did not affect the size of the inhibition zones obtained.

**Tests for bacteriophage activity.** The active antibacterial agent(s) was not transferred from the clear inhibition zones (produced by growing *P. fluorescens* 2-79 on LA for 48 h and overlaying it with *S. enterica* serovar Stanley H0558 as the indicator strain) to freshly prepared lawns of the same indicator strain by either method tested.

**Activity of cell-free supernatant fluids and heat stability of antibacterial factor(s).** Clear zones of inhibition against *S. enterica* serovar Anatum F4317 were evident surrounding the wells containing the cell-free supernatant fluids of *P. fluorescens* 2-79, 2-79LacZ and 2-79.59.34 (average size, 3.2 to 4.5 mm from the edge of the wells) but not 2-79.59.34.24 (no inhibition noted). The GacS mutant *P. fluorescens* 2-79 B46 gave mixed results, with turbid zones of inhibition evident for the first experiment and small clear zones (2.0 cm from the edge of the wells for both experiments) for the second experiment. A few isolated colonies of the indicator strain were evident in all clear zones of inhibition by 24 h of incubation. No change in the appearance of inhibition zones occurred between 24 and 48 h of incubation, except for an increase in the size of the apparent resistant colonies. The heating of cell-free supernatant fluids of *P. fluorescens* 2-79 in a boiling water bath for 10 min before testing did not reduce the size or eliminate the zones of inhibition. No inhibition was noted when *P. fluorescens* 2-79 was used both for the production of cell-free supernatant fluids and as the indicator strain. The growth of *P. fluorescens* 2-79 in LB for 48 h caused an increase in pH from an initial value of 6.8 to a final value of 8.6.

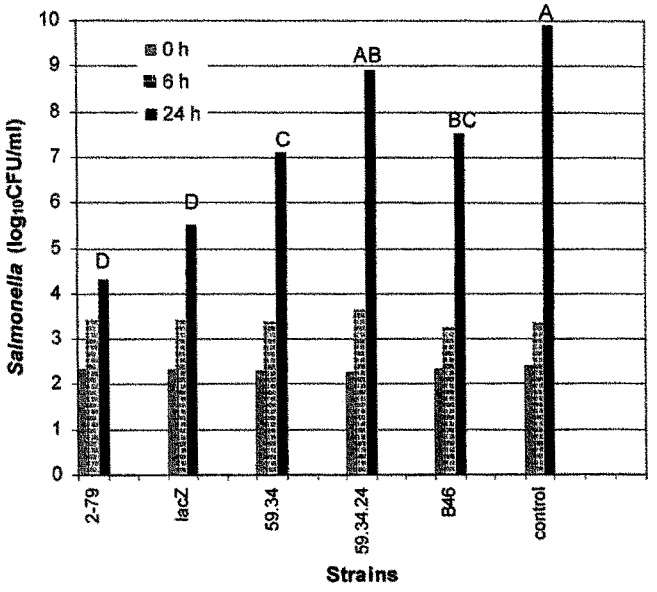


FIGURE 1. *Growth of a five-strain cocktail of* Salmonella enterica *during coculture in tryptic soy broth with* Pseudomonas fluorescens *2-79 and derivative strains at 24°C. Values shown are means of data from two separate experiments. Means (24-h data) with no letters in common were significantly different ($P < 0.05$). There were no significant differences ($P > 0.05$) among bacterial populations at time zero or 6 h.*

**Competitive inhibition in broth culture.** *P. fluorescens* 2-79 and its derivative strains 2-79LacZ, 2-79.59.34, 2-79.59.34.24, and 2-79B46 all exhibited equal growth in TSB, increasing in population by approximately 1 log CFU/ml at 6 h and by approximately 5 log CFU/ml at 24 h to a final population of approximately 10 log CFU/ml (data not shown). No change in media pH occurred after the growth of strain 2-79 alone for 24 h. The results for the growth of a bacterial cocktail consisting of five *S. enterica* strains in the presence of strain 2-79 and its derivatives are shown in Figure 1. No reduction in the population of *S. enterica* at 6 h of incubation was noted during coculture with any of the strains tested. However, population reductions were noted for *S. enterica* at 24 h. For the control, the cocktail of *S. enterica* strains reached approximately 10 log CFU/ml at 24 h, but in the presence of the pseudomonads, the final populations of *S. enterica* ranged from approximately 4 to 9 log CFU/ml. The final populations of *S. enterica* in the presence of strain 2-79 and its derivatives were significantly less than for the control, except for derivative strain 2-79.59.34.24. The mean log reductions of *S. enterica* at 24 h were greatest for strains 2-79 and 2-79LacZ (5.60 ± 0.66 and 4.40 ± 0.15 log CFU/ml, respectively).

**Bioassays on alfalfa sprouts.** Preliminary studies comparing the growth of the parent and the antibiotic-resistant derivatives of the four *S. enterica* serovars in TSB or TSB supplemented with antibiotics with incubation at 37°C (250 rpm), as appropriate, indicated that the derivatives had longer generation times (an average of 0.32 ± 0.04 h for the derivatives versus an average of 0.19 ± 0.00 h for the parent strains). Each of the individual cultures of the antibiotic-resistant derivatives of the four *S. enterica*

TABLE 3. *Reduction of* Salmonella *growth on 6-day-old alfalfa sprouts grown from inoculated seed and treated with a bacterial antagonist*[a]

| Bacterium | Log reduction (CFU/g) | | | |
|---|---|---|---|---|
| | Expt 1 | Expt 2 | Expt 3 | Avg ± SD[b] |
| *Pseudomonas aureofaciens* | | | | |
| 30-84 | 2.32[c] | 0[c] | 0.29 | 0.87 ± 1.26 C |
| 30-84Z | 0.97 | 0 | 0 | 0.32 ± 0.56 C |
| ATCC 13985 | 0.18 | 0.40 | 2.18 | 0.92 ± 1.10 C |
| *P. chlororaphis* | | | | |
| ATCC 9446 | 1.41 | 1.11 | 2.79 | 1.77 ± 0.90 BC |
| *P. fluorescens* | | | | |
| 2-79 | 5.44 | 3.92 | 5.59 | 4.98 ± 0.92 A |
| 2-79LacZ | 5.45 | 4.09 | 5.52 | 5.02 ± 0.81 A |
| 2-79.59.34 | 2.78 | 4.30 | 4.71 | 3.93 ± 1.02 AB |
| 2-79.59.34.24 | 0 | 0 | 1.00 | 0.33 ± 0.58 C |
| 2-79B46 | 0.34 | 1.97 | 1.16 | 1.16 ± 0.81 BC |
| CHA0 | 1.16 | 0[c] | 0[c] | 0.39 ± 0.67 C |
| Pf-5 | 0.29[c] | 0[c] | 0[c] | 0.10 ± 0.17 C |
| Q2-87 | 1.98[c] | 0[c] | 2.44[c] | 1.47 ± 1.30 BC |
| Q8r1-96 | 2.24[c] | 2.40[c] | 0.47[c] | 1.70 ± 1.07 BC |

[a] The population of the cocktail of *Salmonella enterica* strains on sprouts grown from seed not treated with an antagonist averaged 6.50 log CFU/g (range, 5.75 to 7.62 log CFU/g).

[b] Means followed by the same letter are not significantly different ($P > 0.05$).

[c] Evidence of phytotoxicity—poor seed germination or poor growth of sprouts.

serovars used to prepare the cocktail and to inoculate the seed contained approximately 9.6 log CFU/ml. After inoculation and drying, populations of *S. enterica* on the inoculated seed were determined to be 3.9 log CFU/g, on the basis of plating on the selective agar medium XLT-4, and 4.4 log CFU/g, on the basis of plating on TSASN. From the data obtained with TSASN and assuming that there are ca. 500 seeds per g of alfalfa seed, the average inoculum load per seed was approximately 50 CFU.

*P. fluorescens* 2-79 and its derivatives 2-79LacZ and 2-79.59.34 were the most effective inhibitors of the growth of the cocktail of *S. enterica* serovars on sprouting alfalfa seed, averaging reductions of 4 to 5 log CFU/g at day 6 (Table 3). Sprouts grown from contaminated seed treated with these strains appeared normal, and fresh weights of the harvested sprouts grown from treated seed were similar to the controls (data not shown). Treatment with *P. fluorescens* 2-79B46 was less effective, resulting in an average reduction of 1.2 log CFU/g, whereas treatment with derivative 2-79.59.34.24 resulted in no control of *S. enterica* growth in two of the three experiments, with an average reduction for the three experiments of only 0.33 log CFU/g. The other pseudomonads tested were also ineffective antagonists of *S. enterica,* and several were inhibitory toward seed germination and subsequent sprout growth.

Time course experiments demonstrated an increase of approximately 3 log CFU/g for the four-strain cocktail of *S. enterica* within the first 24 h of sprouting for the untreated control (Fig. 2). For the remainder of the 6-day sprouting period, the populations of *S. enterica* on the control sprouts remained stable. In the presence of strain 2-79, populations of *S. enterica* did not increase during the 6-day sprouting period, and populations were significantly lower ($P < 0.05$) than populations of *Salmonella* in the absence of strain 2-79 on days 1, 2, 3, and 6 (Fig. 2). In this set of experiments, the average log reduction at day 6 was approximately 4 log CFU/g.

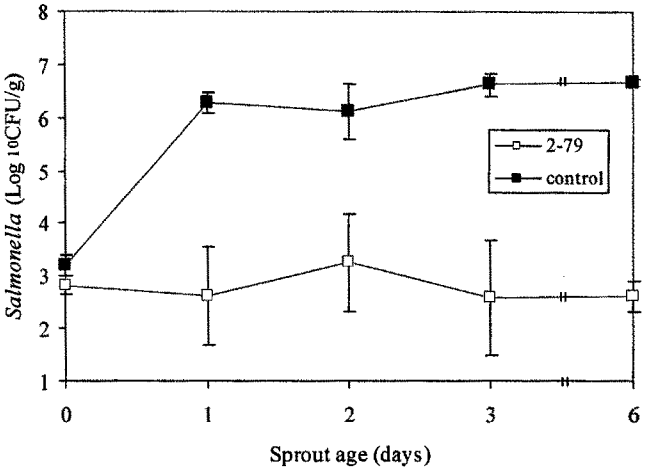


FIGURE 2. *Outgrowth of* S. enterica *from inoculated alfalfa seed during a 6-day sprouting period with or without the addition of* P. fluorescens *2-79 to the seed soak solution prior to seed germination. Values shown represent the means of data from three separate experiments. Populations of* Salmonella *in the presence or absence of strain 2-79 were significantly different* ($P < 0.05$) *at all time points, except for day 0.*

## DISCUSSION

The use of native microflora as biological control agents to control the survival and growth of bacterial human pathogens on alfalfa sprouts may be an alternative to, or may be applied after, chemical and physical interventions, such as the currently recommended seed soak in 20,000 ppm of chlorine *(50),* to further decrease the risk of illness due to contaminated sprouts. When screening the wild-type strains for antibiosis against *Salmonella* on agar media, *P. fluorescens* 2-79 demonstrated the highest degree of antibiosis against *S. enterica. P. fluorescens* 2-79 has been studied for many years as a biological control agent because of its ability to control the plant disease take-all of wheat caused by the fungus *G. graminis* var. *tritici.* The bacterium produces the antifungal compounds PCA and pyoverdine (an iron-binding siderophore), with the production of PCA playing the predominant role in controlling take-all disease *(10, 13, 31, 48).* The additional fluorescent pseudomonads *P. aureofaciens* 30-84 and ATCC 13985 (also producers of PCA)—as well as *P. fluorescens* Pf-5, CHA0, Q2-87, and Q8r1-96 (producers of antifungal compounds, including 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, and hydrogen cyanide) *(28, 34, 36, 53)*—were not inhibitory toward *Salmonella* in the agar spot bioassays. Of these strains, all but *P. aureofaciens* ATCC 13985 have

been reported to be biological control agents with activity against various soilborne plant fungal diseases *(36)*.

In addition to the results of the agar spot bioassays, the results of both the coculture experiments performed in broth and the assays for antibacterial activity of cell-free supernatant fluids indicate that *P. fluorescens* 2-79 produces a diffusible antibacterial substance(s) inhibitory toward *S. enterica*. The nature of the antibacterial substance(s) produced by *P. fluorescens* 2-79 that is inhibitory toward *Salmonella* is not known. Whatever the nature of the substance(s), *P. fluorescens* 2-79 appears resistant to its antimicrobial effects. Several observations indicate that the production of the iron-binding fluorescent siderophore (pyoverdine) by strain *P. fluorescens* 2-79 was not responsible for antibacterial activity. First, growth on the low-iron medium PAF (a medium that favors the production of siderophore *(21)*) did not favor antibiosis. Second, the addition of iron to PAF led to an increase, rather than a decrease, in antibiosis. Third, the siderophore-deficient derivative *P. fluorescens* 2-79.59.34 was not compromised in antibiosis, as demonstrated in both in vitro and in situ bioassays. This is in contrast to the results of two earlier studies, which indicated a role for siderophore production in the inhibition of growth of *S. enterica* serovar Typhimurium and *S. enterica* subsp. *arizoniae* on agar media by *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis (9, 22)*.

The production of PCA also does not appear to be responsible for the antibiosis exhibited by *P. fluorescens* 2-79 against *S. enterica*. The PCA-deficient derivative *P. fluorescens* 2-79LacZ was not reduced in activity in vitro or in situ, and purified PCA did not inhibit the growth of *S. enterica* serovar Anatum F4317 when tested at up to 100 μg in the disk diffusion assay. In addition, the other PCA-producing bacteria *P. aureofaciens* 30-84 and ATCC 13985 tested were not effective antagonists either in vitro or in situ. The in vitro inhibitory activity of PCA has been reported to be greater against fungi than against bacteria *(10)*.

The antibacterial substance(s) produced by *P. fluorescens* 2-79 that is inhibitory toward the growth of *S. enterica* appears to be under the regulatory control of the two-component system GacS-GacA, as evidenced by the lack of significant in vitro and in situ activity of the two GacS mutants tested (*P. fluorescens* 2-79.59.34.24 and 2-79B46). GacS is the sensor kinase, and GacA is the response regulator of a two-component regulatory system that exerts a positive effect on the production of a variety of extracellular metabolites *(12)*. *Pseudomonas* spp. with mutations in the *gacS-gacA* regulatory region are deficient in the production of antibiotics as well as extracellular enzymes, including proteases and lipases, and are ineffective in plant disease control *(11, 12)*.

Because of the absence of an inhibitory effect of heat treatments, the antibiosis exhibited by *P. fluorescens* 2-79 also does not appear to be the result of the production of proteases, lipases, or heat-labile bacteriocins. The involvement of bacteriophages appears unlikely because of the inability to transfer the antibacterial substance(s) from clear zones of inhibition to freshly prepared lawns of a susceptible indicator strain by two different methods. Localized acidification of the media by *P. fluorescens* 2-79 was not involved in antibiosis, as evidenced by no effect of buffering of LA in the agar spot bioassay study. The addition of iron and glucose to the high-iron-containing medium LA reduced the level of antibiosis, whereas the addition of iron to the iron-deficient medium PAF increased antibiosis. Iron may be required in moderate amounts for the optimal production or activity of the active metabolite(s) but, above a certain level, may also be inhibitory. Increases in the production of antifungal metabolites by the addition of iron and glucose to the growth medium have been reported for fluorescent pseudomonads *(6, 37, 40, 48, 52, 53)*.

The growth dynamics of the cocktail of *S. enterica* strains during the sprouting of untreated seed were similar to those previously reported for laboratory-inoculated alfalfa seed *(14, 19)*. The relative efficacy of the fluorescent pseudomonads as antagonists during the sprouting process correlated well with the results of the agar spot bioassays (deferred method) carried out on LA for all bacterial strains, with the exception of *P. chlororaphis* ATCC 9446. This strain demonstrated a greater degree of antagonism in situ than would have been predicted by the in vitro bioassay results. *P. fluorescens* 2-79, 2-79.59.34 (deficient in pyoverdine), and 2-79LacZ (deficient in PCA) were all effective inhibitors of *S. enterica* outgrowth in situ, and no detrimental effects on seed germination or sprout yield and appearance were observed for the wild-type strain, which corresponds to the results reported by Matos and Garland *(25)* for *P. fluorescens* 2-79. The lack of soft-rot symptoms on sprouts grown from treated seed is in agreement with the nonpectolytic nature of *P. fluorescens* 2-79. The observation that *P. fluorescens* 2-79 is an effective biological control strain against *Salmonella* on sprouting alfalfa seed confirms the results of a recent study by Matos and Garland *(25)*. However, the drop-off in efficacy in the control of *Salmonella* outgrowth between days 3 and 7 of sprouting that they reported was not observed through day 6 of the sprouting process in our experiments.

The overall level of *Salmonella* on alfalfa seed in naturally contaminated lots is usually less than 2 per 100 g of seed by the most-probable-number technique *(16)*, which is several orders of magnitude lower than that used in our bioassays (initial population, 4.4 log CFU/g). However, prior results in our laboratory on the dose of gamma irradiation required to kill *S. enterica* in a naturally contaminated seed lot indicated that the population on an individual contaminated seed may reach a level of 4 log CFU *(45)*. That *P. fluorescens* 2-79 strongly inhibited outgrowth of the initial high populations of *S. enterica* present on the seed in our study demonstrates the potential of this strain as a biological control agent under very stringent experimental conditions.

The current study and the previous study of Matos and Garland *(25)* indicate that biological control may be a viable intervention for controlling *S. enterica* outgrowth from contaminated seed during the sprouting process. Matos and Garland *(25)* reported good control of *S. enterica* outgrowth on sprouting alfalfa seed, not only by *P. fluorescens* 2-79 at days 1 and 3 of sprouting (reductions of 4 log CFU/g)

but also by undefined whole microbial communities isolated from market sprouts (a reduction greater than 5 log CFU/g at day 7).

Fluorescent pseudomonads have previously been reported to inhibit the growth of bacterial human pathogens on plant surfaces. The saprophyte *Pseudomonas syringae* inhibited the growth of *E. coli* O157:H7 inoculated into wounds of apple fruit *(17).* Two fluorescent pseudomonads isolated from the leaves of endive significantly inhibited the growth of *Listeria monocytogenes* on endive leaves when inoculated prior to the pathogen *(4).* A fluorescent pseudomonad in combination with a yeast, when coinoculated onto pepper disks, inhibited the growth of *L. monocytogenes (24).*

Further research to identify the heat-stable extracellular antibacterial metabolite(s) responsible for the antibiosis of *P. fluorescens* 2-79 against *S. enterica* is currently under way. Identification of the mechanism(s) of antibiosis may allow the generation of more effective mutants by genetic manipulation. Because of the occurrence of a naturally occurring resistant subpopulation of *S. enterica,* as demonstrated in this study, *P. fluorescens* 2-79 might be best used in a mixture of biological control agents with differing modes of action. Future studies will focus on larger-scale experiments testing strain 2-79 for the control of pathogen outgrowth from contaminated seed under conditions that more closely mimic commercial production (e.g., frequent overhead watering).

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